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(54) **DOUBLE NETWORK BIOINKS**

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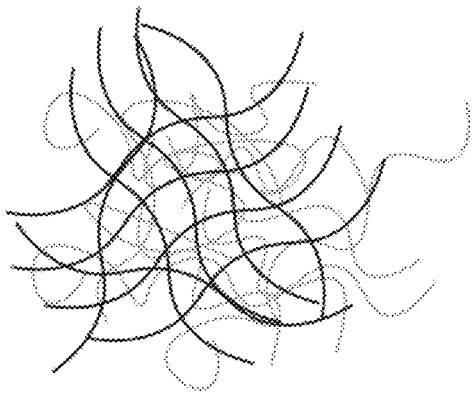
(57) **ABSTRACT**

The present invention relates to a double network bioink that is customizable and tailorable in terms of mechanical characteristics, diffusivity, and biological functionality that is printable at room temperature and cytocompatible. Double network bioinks of the present invention can be utilized as a standard for comparison and evaluation of bioinks and during calibration and/or are suited for the education field as a "blank slate" bioink that can be utilized to convey different scientific concepts. Specific bioinks included comprise: one or more biocompatible or non-biocompatible thickener; one or more polyethylene glycol based crosslinkable network; one or more photoinitiator; and/or optionally, one or more additives to impart desired and/or different characteristics to the bioink. In embodiments the thickener and polyethylene glycol based crosslinkable network form a structure comprising two interpenetrating networks.

25 Claims, 4 Drawing Sheets



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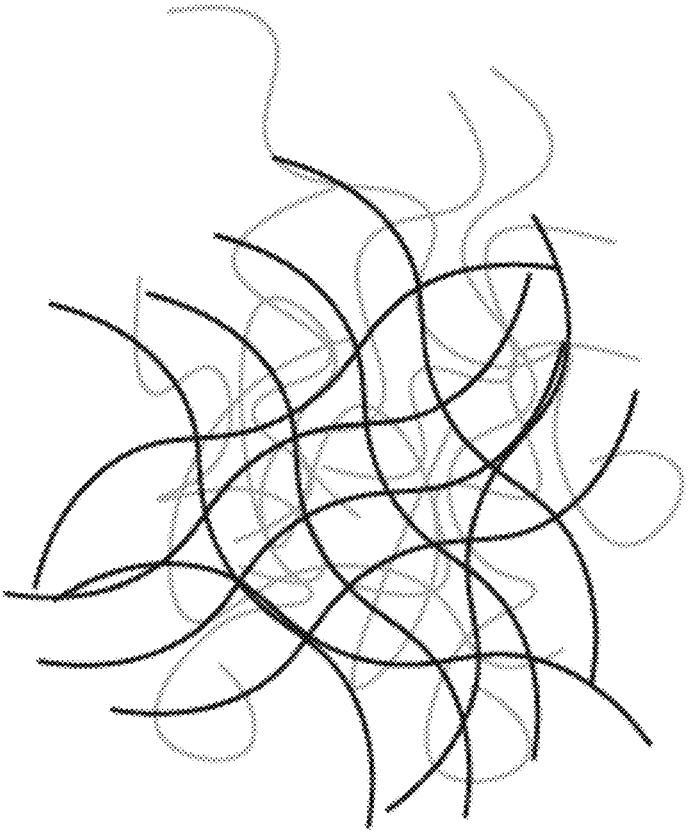
Halper, J.; Kjaer, M. (2014) Chapter 3: Progress in Heritable Soft Connective Tissue Diseases. Advances in Experimental Medicine and Biology, vol. 802, 26 pages.

(56) **References Cited**

OTHER PUBLICATIONS

Kumar, A. et al. Carbohydrate Polymers, "Application of xanthan gum as polysaccharide in tissue engineering: A review", published online Oct. 5, 2017, vol. 180 pp. 128-144.

* cited by examiner



CROSSLINKED

FIG. 1B

UN-CROSSLINKED

FIG. 1A

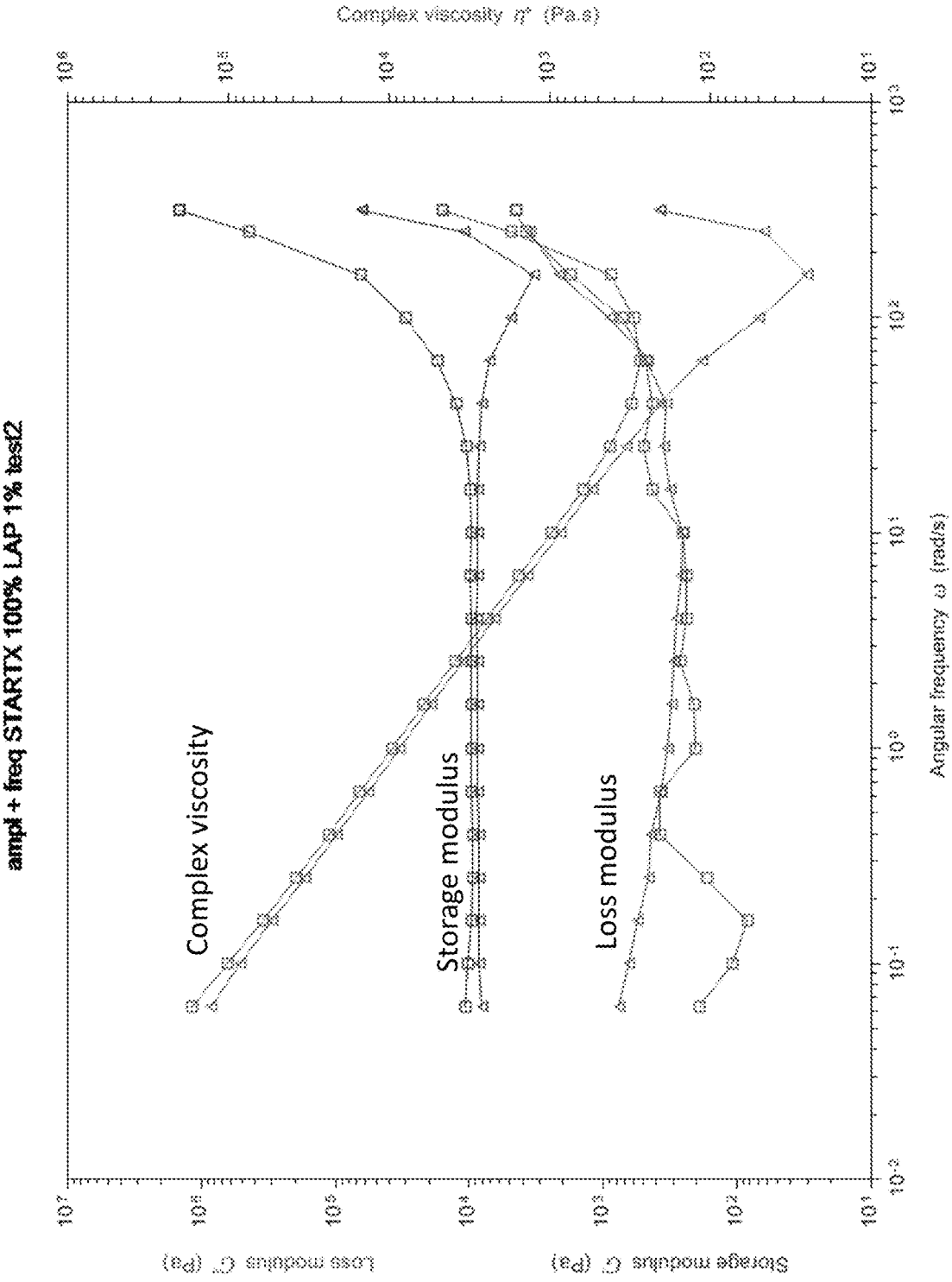

ampl + freq STARTX 100% LAP 1% test2
Complex viscosity
Storage modulus
Loss modulus
Complex viscosity η* (Pa.s)
Storage modulus G' (Pa)
Loss modulus G'' (Pa)
Angular frequency ω (rad/s)

FIG. 2

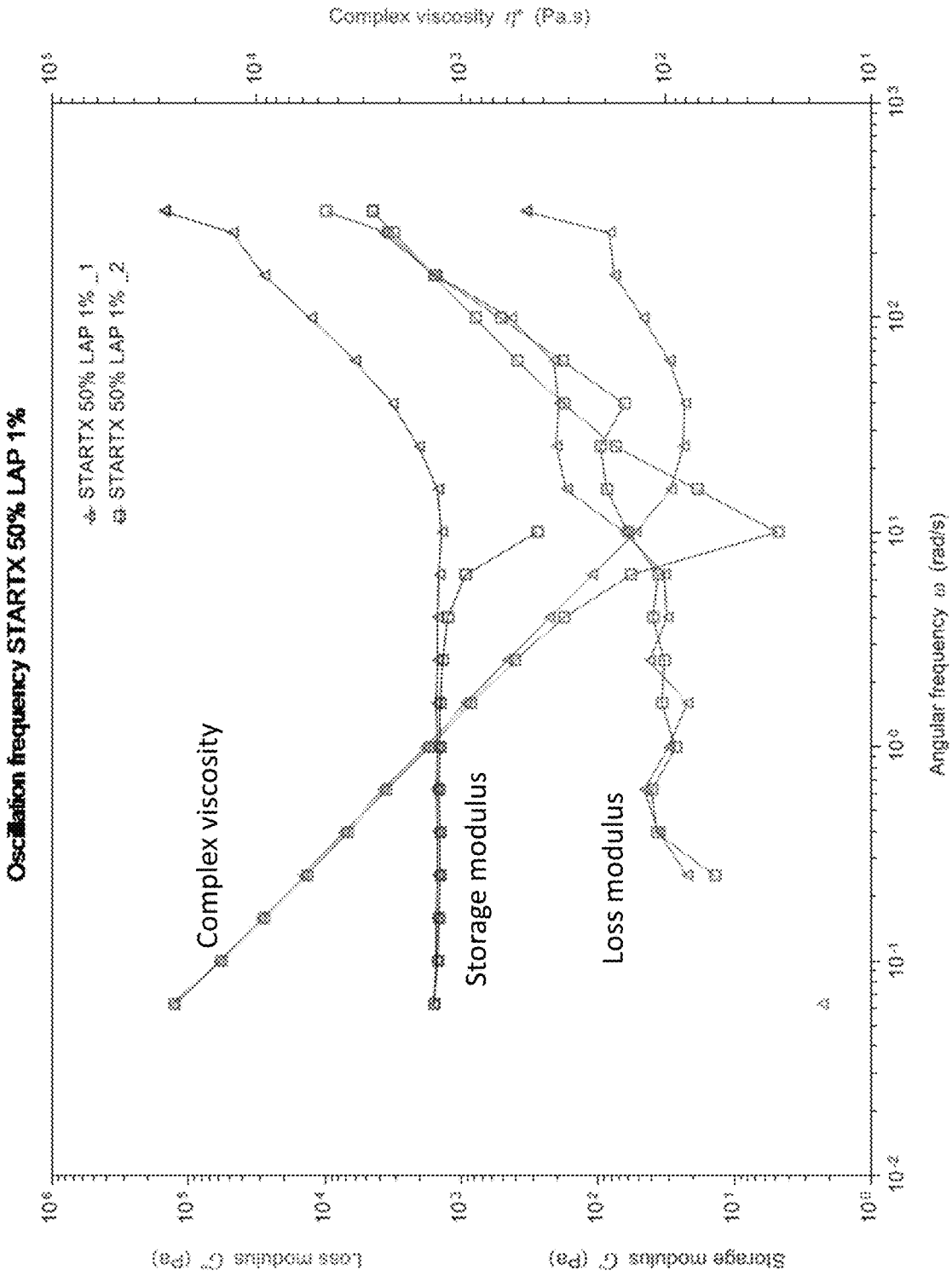

Oscillation frequency STARTX 50% LAP 1%
STARTX 50% LAP 1% _1
STARTX 50% LAP 1% _2
Complex viscosity
Storage modulus
Loss modulus
Complex viscosity η* (Pa.s)
Storage modulus G' (Pa)
Loss modulus G'' (Pa)
Angular frequency ω (rad/s)

FIG. 3

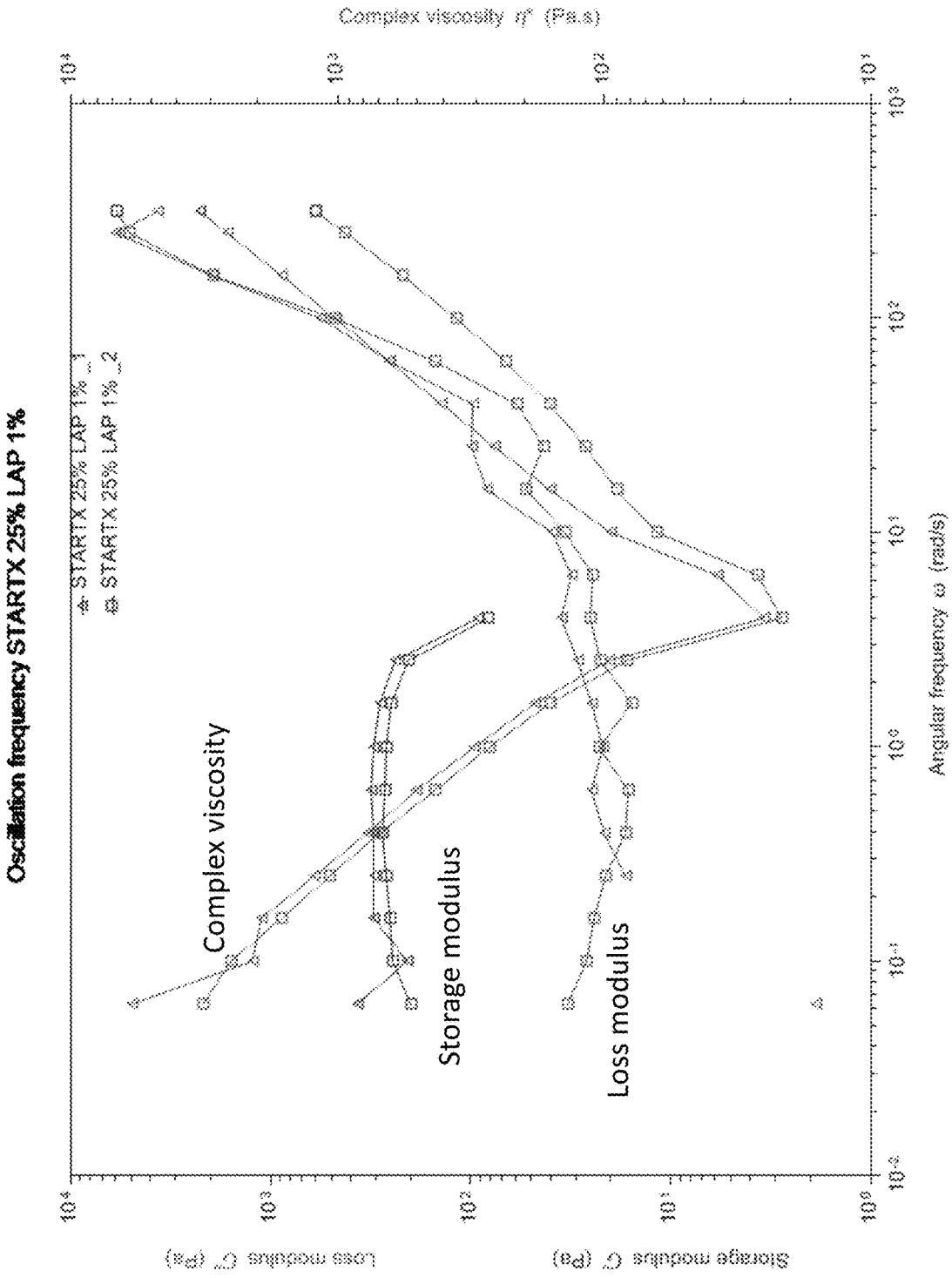

Oscillation frequency STARTX 25% LAP 1%
STARTX 25% LAP 1%_1
STARTX 25% LAP 1%_2
Complex viscosity
Storage modulus
Loss modulus
Complex viscosity η* (Pa.s)
Angular frequency ω (rad/s)
Storage modulus G' (Pa)
Loss modulus G'' (Pa)

FIG. 4

DOUBLE NETWORK BIOINKS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a National Stage application under 35 U.S.C. 371 of International Application No. PCT/US19/55684, filed Oct. 10, 2019, which application claims priority to and the benefit of the filing date of U.S. Provisional Application Nos. 62/744,034, filed Oct. 10, 2018 and 62/748,948, filed Oct. 22, 2018. The disclosures of each of these applications are hereby incorporated by reference herein in their entireties.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a double network bioink that is customizable and tailorable in terms of mechanical characteristics, diffusivity, and biological functionality that is printable at room temperature and cytocompatible.

The present invention also relates to a double network bioink that can be utilized as a standard for comparison and evaluation of bioinks and during calibration.

The present invention also relates to a double network bioink that is suited for the education field as a 'blank slate' bioink that can be utilized to convey different scientific concepts.

SUMMARY OF THE INVENTION

Embodiments of the present invention provide a bioink that is comprised of a double network hydrogel. The double network includes a non-crosslinkable thickener and a cross-linkable polyethylene glycol-based network that interpenetrate each other The bioink is a blank slate in terms of customization. Furthermore, the resulting mechanical properties, diffusivity, and biological properties can be tailored through addition of pendant groups that can link to the polyethylene glycol-based network.

Specific embodiments include Embodiment 1, which is a bioink comprising: one or more biocompatible or non-biocompatible thickener; one or more polyethylene glycol based crosslinkable network; one or more photoinitiator; and/or optionally, additives to impart one or more desired or different characteristics; wherein the thickener and polyethylene glycol based crosslinkable network form a structure comprising two interpenetrating networks.

Such embodiments include Embodiment 2, which is the bioink of Embodiment 1, wherein the thickener comprises one or more of: polyethylene oxide; polypropylene oxide; nanofibrillar cellulose; nanocrystalline cellulose; gelatin; collagen; glucomannon; alginate; k-carrageenan; bentonite clay; and/or xanthan gum.

Embodiment 3 is the bioink of Embodiments 1 or 2, where the thickener comprises polyethylene oxide having an average molecular weight of: 100,000 Daltons; 200,000 Daltons; 300,000 Daltons; 400,000 Daltons; 600,000 Daltons; 900,000 Daltons; 1,000,000 Daltons; 2,000,000 Daltons; 4,000,000 Daltons; 5,000,000 Daltons; or 8,000,000 Daltons, or any range or value encompassed by these values.

Embodiment 4 is the bioink of any preceding Embodiment, wherein the gelatin comprises a Bloom Strength of: 150; 175; 200; 225; 250; 275; or 300.

Embodiment 5 is the bioink of any preceding Embodiment, wherein the thickener comprises alginate with

molecular weights of: low molecular weight (average below 75 kDa); medium molecular weight (average between 75 and 200 kDa); or high molecular weight (average above 200 kDa).

Embodiment 6 is the bioink of any preceding Embodiment, wherein the thickener comprises alginate with a guluronic acid (G)/mannuronic acid (M) ratio of: <1 G/M; 1 G/M; or >1.5 G/M.

Embodiment 7 is the bioink of any preceding Embodiment, wherein the thickener comprises collagens comprised of: acid soluble collagen; precipitated collagen; and/or pepsin digested collagen.

Embodiment 8 is the bioink of any preceding Embodiment, wherein the cross-linkable polyethylene glycol comprises reactive groups comprising: acrylate; thiol; maleimide; and/or biotin.

Embodiment 9 is the bioink of any preceding Embodiment, wherein the polyethylene glycol crosslinker has a molecular weight of: 660 Daltons; 1000 Daltons; 2000 Daltons; 3400 Daltons; 5000 Daltons; 10000 Daltons; 20000 Daltons; or 35000 Daltons.

Embodiment 10 is the bioink of any preceding Embodiment, wherein the polyethylene glycol crosslinker exhibits structures or blends comprising: linear; branched; 4-arm; 8-arm; and/or hyperbranched.

Embodiment 11 is the bioink of any preceding Embodiment, wherein the additives comprise monoacrylate PEG with functionalization of the following: fluorescent groups such as: fluorescein; rhodamine; or dansyl; sulfonate groups; amine groups; phosphate groups; lipid groups; and/or CNT binding.

Embodiment 12 is the bioink of any preceding Embodiment, comprising mPEG-PEG-Phosphate groups which comprise an average molecular weight of: 1000 Daltons; 2000 Daltons; 5000 Daltons; 10000 Daltons; 20000 Daltons; 30000 Daltons; or 40000 Daltons, or any range or value encompassed by these values.

Embodiment 13 is the bioink of any preceding Embodiment, wherein the photoinitiator comprises: Irgacure 2959; LAP; Eosin-Y; and/or Avidin.

Embodiment 14 is a method of forming a double network structure comprising: providing a bioink, wherein the bioink comprises: one or more thickener, one or more cross-linkable polymer, one or more photoinitiator, and optionally comprising one or more additives, and subjecting the bioink to one or more light sources (such as a laser) to induce the cross-linkable polymer to form a first network in the presence of the thickener, which thickener provides a second non-crosslinkable network, wherein together the first and second networks are interpenetrating networks providing a double network structure.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings illustrate certain aspects of some embodiments of the invention, and should not be used to limit or define the invention. Together with the written description, the drawings serve to explain and illustrate certain principles of the invention.

FIG. **1**A is a schematic showing a structure of an uncross-linked bioink.

FIG. **1**B is a schematic showing components of a cross-linked bioink.

FIGS. **2-4** are graphs showing viscosity, storage modulus, and loss modulus for representative bioink compositions made according to embodiments of the invention.

DETAILED DESCRIPTION OF VARIOUS EMBODIMENTS OF THE INVENTION

Reference will now be made in detail to various exemplary embodiments of the invention. It is to be understood that the following discussion of exemplary embodiments is not intended as a limitation on the invention. Rather, the following discussion is provided to give the reader a more detailed understanding of certain aspects and features of the invention.

Definitions

The following definitions are provided to facilitate understanding of certain terms provided in this specification. For other terms not defined herein, the ordinary meaning as recognized by an ordinarily-skilled artisan should be applied.

Bioink: a biomaterial that can be used in its present state or has been adapted for use within a 3D bioprinter system. Bioinks must both support cell viability and maintain structural support during and after the deposition process.

Bioprinting: the utilization of 3D printing and 3D printing-like techniques to combine cells, growth factors, and biomaterials to fabricate biomedical parts that maximally imitate natural tissue characteristics.

Bioink Standard: a bioink that has standardized characteristics for comparison to and evaluation of different bioinks. Standardized characteristics can include concentrations, swelling ratio, hydrophobicity, compressive strength, elastic strength, viscoelastic behavior, gelation point, degradation, color, crosslinking speed.

Swelling Ratio: Volume increase of a biomaterial construct after it is submerged in a water bath, saline solution, or cell media.

Hydrophobicity: The tendency of a biomaterial to repel or absorb water.

Compressive Strength: The resistance of a bioink or construct to compression.

Elastic Strength: The stiffness of the bioink under linear deformation.

Gelation Point: the point by which a biomaterial begins to possess storage modulus whether through chain entanglement or self-assembly.

Chain Entanglement: A mechanism of gelation by which chains form physical entanglements with each other, often driven by temperature.

Self-Assembly: the process by which a material assembles into a different state under the right thermal and energetic conditions.

Opacity: the transmission of light through the biomaterial.

Cross-linking: The process by with a biomaterial changes from a non-permanent state of gelation to a permanent state through the introduction of a reactive species to form physical cross-links between adjacent polymer chains

Kinetics: The rate by which a cross-linking reaction proceeds.

Degradation: the physical, chemical, and/or enzymatic deterioration of a biomaterial into its byproducts. Degradation is dependent on the physical and chemical characteristic of the biomaterial.

Calibration Standard: A bioink that is utilized to establish or verify parameters to prepare a bioprinter for bioprinting.

Diffusivity: A characteristic of a hydrogel material based on the rate that a molecule moves through the network. Diffusivity is dependent on the characteristics of the network itself, including mesh size, surface charge, presence of binding groups.

Double Network: A material that includes two interpenetrating networks. The bulk of the material is known as a thickener. This thickener is non-crosslinked and provides structure to the network. The second network is the cross-linkable network that provides rigidity to the structure. A double network can include a plurality of networks, but preferably two, and with at least one network being a non-crosslinkable network and at least one network being a cross-linkable network.

Thickener: A non-crosslinkable natural or synthetic polymer network that can exhibit shear-thinning properties, or increase viscosity of a blend. If not blended with a cross-linked network, the thickener material will be diluted away.

Network Preparation Methods: The inventive bioinks are prepared by combining/mixing the components under conditions that avoid formation of the double network until desired. The bioinks can be used to form structures (such as biocompatible structures and/or scaffolds) comprising a plurality of networks. Preferably, the bioinks can be used to form double networks. To make a double network two or more polymer precursor solutions are mixed together. The precursor solutions are selected such that one network will crosslink or entangle at a different time than the other components. For example, a PEGDA precursor solution is mixed with a non-crosslinkable thickener that forms a physical network. During the mixing process both networks can be blended together. A photoinitiator is also blended into this material. This blend can then be crosslinked under UV light to crosslink the PEGDA precursor solution and 'lock in' the thickener network (FIGS. **1A-1B**). This creates the double network. A double network can also be created by using a crosslinkable network that self-assembles under heat or normal molecular interactions. Methods of forming double networks can also include providing a bioink comprising one or more thickener, one or more cross-linkable polymer, one or more photoinitiator, and optionally comprising one or more additives, and subjecting the bioink to one or more light sources (such as a laser) to induce the cross-linkable polymer to form a first network in the presence of the thickener, which thickener forms/provides a second non-crosslinkable network, and together the first and second networks are interpenetrating networks providing a double network structure. To facilitate formation of either or both of the first and second networks, the components can be allowed to interact at a temperature ranging from 0-180 degrees C., such as at room temperature for a period of 5 seconds to 24 hours or more. In embodiments, the bioink can comprise as the cross-linkable polymer a polyethylene glycol based crosslinkable polymer and as the thickener one or more of polyethylene oxide, polypropylene oxide, nanofibrillar cellulose, nanocrystalline cellulose, gelatin, collagen, glucomannon, alginate, k-carrageenan, bentonite clay, and/or xanthan gum.

Educational Bioink: A bioink that is developed for the purpose of conveying a scientific concept.

Blank Slate: A bioink that is inert in its base functionality but can be easily customized toward different applications based on one or more additives.

EXAMPLES/APPLICATIONS

FIGS. **2-4** show various characteristics for bioink compositions made according to embodiments of the invention. The results show that the mechanical stiffness of the bioink

can be tailored by changing its composition while not effecting its printability. This allows different standardized constructs to be made that can exhibit distinct mechanical characteristics. This tailoring allows constructs to be exposed to distinct mechanical environments. Additionally, it allows the development of more stable support structures that match the properties of the bioprinted constructs better. This eliminates the chance that a mechanical gradient can develop that will affect cell behavior negatively.

An exemplary bioink composition according to embodiments of the invention can include (a) one or more thickener present in an amount ranging from 0.1% to 20% w/v of the composition, (b) one or more PEGDA crosslinker present in an amount ranging from 0.1% to 10% w/v of the composition, (c) one or more photoinitiator present in an amount ranging from 0.05% to 1% w/v of the composition, and (d) optionally one or more supplementary proteins present in an amount ranging from 0.01% to 10% w/v of the composition, with the remainder comprising water and salts. By varying the type and/or amount of any one or more of the thickener, PEGDA crosslinker and/or photoinitiator, the mechanical stiffness, the diffusivity, the elasticity, binding capacity, etc. of the construct can be varied. An application of embodiments of this bioink is in the field of surgical models. The elasticity and stiffness of the cross-linked bioink can be controlled by changing the blending ratio between the thickener, cross-linking polymer, and the concentration of the photoinitiator.

Embodiments of the double network bioink can be utilized as a base material for surgical models. These surgical models can be customized and made more complex through the use of specialized double network bioinks. In order to fabricate a surgical model, variations of the double network bioink that exhibit different mechanical characteristics can be utilized for different tissues. Additionally, sacrificial bioinks such as pluronics can be used.

Embodiments of the double network bioink can be utilized as a base material to physically or chemically secure a bioprinted construct to a surface. A variation of this double network bioink can lack the necessary components that facilitate the adhesion or migration of cells. Additionally, the double network bioink can be partially crosslinked or non-crosslinked when a bioprinted construct is placed or printed onto the surface. Polymer molecules from the double network bioink can directly entangle with the bioprinted construct. Additionally, if a bioink is utilized that contains cross-linkable groups, the bioprinted construct and the double network bioink can be directly linked together.

This Embodiments of the double network bioink can be utilized as a tailorable surface that a bioprinted construct can be deposited onto. Many cells within fibrillar hydrogel networks can sense mechanical forces of surrounding boundary points and surfaces. This is also a problem within the broader 3D cell culture and bioprinting field where cells within a construct will migrate to the tissue culture plate surface and begin to spread out from the construct. This creates boundary and edge irregularities in cell growth. The double network bioink can be bioprinted with controlled mechanical stiffness. When combined with a tissue construct, the double network ink can mask the stiffness of the well plate surface by matching the mechanical characteristics of the constructs so as to not create a gradient. Furthermore, the mechanical characteristics can be controlled to drive cell migration, if desired. Possible ratios of mechanical stiffness of the double network bioink to the construct include: 100:1, 10:1, 5:1, 1:1, 1:5, 1:10, and 1:100.

Embodiments of the double network bioink can be utilized as an elastic and pliable support material that can transmit forces and other applied stimuli to bioprinted construct via a bioreactor system. The double network hydrogel can be bioprinted into the lattice structure of a bioprinted construct and crosslinked, then can be utilized to apply mechanical stimulation such as tension and compression onto the bioprinted lattice.

Embodiments of the double network can be utilized as a reservoir for growth factor release. Through functionalization with growth factor or other small molecule binding groups such as sulfonated groups, heparins, peptides, and DNA/RNA, the bioink can be tailored to bind, sequester, and release growth factors and other molecules.

Embodiments of the double network bioink can be utilized as a component of organ-on-a-chip platforms. The bioink can be utilized as a novel material for utilization in organ-on-a-chip systems as an alternative to traditional polydimethylsiloxane (PDMS). This double network bioink can support the diffusion of nutrients and small molecules across barriers in an organ-on-a-chip system. Additionally, the bioink can be bioprinted and cross-linked concurrently with other bioinks, enabling the fabrication of an organ-on-a-chip system without the need for curing under heat or other harsh conditions found with traditional chip silicones.

Embodiments of the double network bioink can be utilized as a standardized bioink to enable mechanical comparisons between bioink materials.

Embodiments of the double network can be utilized as a prefabricated support structure such a well, trough, ring mold, or other material that can be combined with another biomaterial to fabricate scaffolding materials.

The present invention has been described with reference to particular embodiments having various features. It will be apparent to those skilled in the art that various modifications and variations can be made in the practice of the present invention without departing from the scope or spirit of the invention. One skilled in the art will recognize that these features may be used singularly or in any combination based on the requirements and specifications of a given application or design. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention. Where the term "comprising" is used, it should be understood that the disclosures equally include such embodiments "consisting essentially of" or "consisting of" the details recited as well. Where a range of values is provided in this specification, each value between the upper and lower limits of that range is also specifically disclosed. The upper and lower limits of these smaller ranges may independently be included or excluded in the range as well. As used in this specification, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. It is intended that the specification and examples be considered as exemplary in nature and that variations that do not depart from the essence of the invention are intended to be within the scope of the invention. Further, the references cited in this disclosure are incorporated by reference herein in their entireties.

The invention claimed is:

1. A bioink comprising:

(a) a biocompatible or non-biocompatible thickener;

(b) a polyethylene glycol based crosslinkable network;

(c) a photoinitiator; and/or

(d) optionally, additives to impart different characteristics, wherein the additives comprise monoacrylate PEG with functionalization of the following:

fluorescent groups such as:
i. fluorescein;
ii. rhodamine; or
iii. dansyl
sulfonate groups;
amine groups;
phosphate groups;
lipid groups; and/or
CNT binding;
wherein the thickener and polyethylene glycol based crosslinkable network form a structure comprising two interpenetrating networks; and
wherein the thickener comprises polyethylene oxide having an average molecular weight of from 100,000 Daltons to 8,000,000 Daltons.

2. The bioink of claim **1**, wherein the thickener further comprises one or more of polyethylene oxide; polypropylene oxide; nanofibrillar cellulose; nanocrystalline cellulose; gelatin; collagen; glucomannon; alginate; k-carrageenan; bentonite clay; and/or xanthan gum.

3. The bioink of claim **2**, wherein the gelatin comprises a bloom strength ranging from 150 to 300.

4. The bioink of claim **1**, wherein the thickener further comprises alginate with a molecular weight average of below 75 kDa.

5. The bioink of claim **1**, wherein the thickener further comprises alginate with a guluronic acid (G)/mannuronic acid (M) ratio of <1 G/M.

6. The bioink of claim **1**, wherein the thickener further comprises collagens comprised of:
(a) Acid soluble collagen;
(b) Precipitated collagen; and/or
(c) Pepsin digested collagen.

7. The bioink of claim **1**, wherein the cross-linkable polyethylene glycol comprises reactive groups comprising:
(a) Acrylate;
(b) Thiol;
(c) Maleimide; and/or
(d) Biotin.

8. The bioink of claim **1**, wherein the cross-linkable polyethylene glycol comprises a linear; branched; 4-arm; 8-arm; and/or hyperbranched structure.

9. The bioink of claim **1**, wherein the thickener is present in an amount ranging from 0.1% to 20% w/v of the composition.

10. The bioink of claim **1**, wherein the cross-linkable polyethylene glycol is PEGDA present in an amount ranging from 0.1% to 10% w/v of the composition.

11. The bioink of claim **1**, wherein the photoinitiator is present in an amount ranging from 0.05% to 1% w/v of the composition.

12. The bioink of claim **1**, wherein the photoinitiator comprises Irgacure 2959; LAP; Eosin-Y; and/or Avidin.

13. A bioink comprising:
a biocompatible or non-biocompatible thickener;
a polyethylene glycol based crosslinkable network;
a photoinitiator; and/or
optionally, additives to impart different characteristics;
wherein the thickener and polyethylene glycol based crosslinkable network form a structure comprising two interpenetrating networks; and
further comprising mPEG-PEG-Phosphate groups, which comprise an average molecular weight of 1000 Daltons; 2000 Daltons; 5000 Daltons; 10000 Daltons; 20000 Daltons; 30000 Daltons; or 40000 Daltons.

14. The bioink of claim **13**, wherein the thickener comprises one or more of polyethylene oxide; polypropylene oxide; nanofibrillar cellulose; nanocrystalline cellulose; gelatin; collagen; glucomannon; alginate; k-carrageenan; bentonite clay; and/or xanthan gum.

15. The bioink of claim **13**, wherein the thickener comprises polyethylene oxide having an average molecular weight ranging from 100,000 Daltons to 8,000,000 Daltons.

16. The bioink of claim **14**, wherein the gelatin comprises a bloom strength ranging from 150 to 300.

17. The bioink of claim **13**, wherein the thickener comprises alginate with an average molecular weight of below 75 kDa.

18. The bioink of claim **13**, wherein the polyethylene glycol based crosslinkable network comprises cross-linkable polyethylene glycol comprising one or more reactive groups chosen from acrylate; thiol; maleimide; and/or biotin.

19. The bioink of claim **1**, wherein the thickener further comprises alginate with a molecular weight average of between 75 and 200 kDa.

20. The bioink of claim **1**, wherein the thickener further comprises alginate with a molecular weight average of above 200 kDa.

21. The bioink of claim **1**, wherein the thickener further comprises alginate with a guluronic acid (G)/mannuronic acid (M) ratio of 1 G/M.

22. The bioink of claim **1**, wherein the thickener further comprises alginate with a guluronic acid (G)/mannuronic acid (M) ratio of >1.5 G/M.

23. A bioink comprising:
(a) a biocompatible or non-biocompatible thickener;
(b) a polyethylene glycol based crosslinkable network;
(c) a photoinitiator; and/or
(d) optionally, additives to impart different characteristics, wherein the additives comprise monoacrylate PEG with functionalization of the following:
fluorescent groups such as:
i. fluorescein;
ii. rhodamine; or
iii. dansyl
sulfonate groups;
amine groups;
phosphate groups;
lipid groups; and/or
CNT binding;
wherein the thickener and polyethylene glycol based crosslinkable network form a structure comprising two interpenetrating networks; and
wherein the polyethylene glycol based crosslinkable network comprises cross-linkable polyethylene glycol having an average molecular weight ranging from 660 Daltons to 35000 Daltons.

24. The bioink of claim **13**, wherein the thickener comprises alginate with an average molecular weight of between 75 and 200 kDa.

25. The bioink of claim **13**, wherein the thickener comprises alginate with an average molecular weight of above 200 kDa.

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